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Characterization of *Gh*Rac1 GTPase expressed in developing cotton (*Gossypium hirsutum* L.) fibers

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Abstract

Cytoskeleton assembly plays an important role in determining cotton fiber cell length and morphology and is developmentally regulated. As in other plant cells, it is not clear how cytoskeletal assembly in fibers is regulated. Recently, several Rac/Rop GTPases in *Arabidopsis* were shown to regulate isotropic and polar cell growth of root hairs and pollen tubes by controlling assembly of the cytoskeleton. *GhRac1*, isolated from cottonseeds, is a member of the Rac/Rop GTPase family and is abundantly expressed in rapidly growing cotton tissues. *GhRac1* shows the greatest sequence similarity to the group IV subfamily of *Arabidopsis* Rac/Rop genes. Overexpression of *GhRac1* in *E. coli* led to the production of a functional GTPase as shown by in vitro enzyme activity assay. In contrast to other Rac/Rop GTPases found in cotton fiber, *GhRac1* is highly expressed during the elongation stage of fiber development with expression decreasing dramatically when the rate of fiber elongation declines. The association of highest *GhRac1* expression during stages of maximal cotton fiber elongation suggests that *Gh*Rac1 GTPase may be a potential regulator of fiber elongation by controlling cytoskeletal assembly.
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Keywords: Cytoskeleton; *Gossypium hirsutum*; GTPase; Rac; Real-time RT-PCR; Rop

1. Introduction

Small GTPases, monomeric guanine nucleotide binding proteins in eukaryotic cells, are molecular switches [1,2] becoming activated by GTP, and inactivated by the hydrolysis of GTP to GDP. The small-GTPase superfamily of *Arabidopsis* consists of Rab, Ran, Arf, and Rho GTPases. Rho-family GTPases regulate the actin cytoskeleton in yeast and animal cells [3]. Animal Rho GTPases are classified into three subfamilies, Rho, Rac and Cdc42 [2]. In plants, Rac or Rac-like GTPases were identified based on sequence similarity with human Rac GTPases [4–6]. Since plant, fungal and animal Rho and Rac GTPases do have some sequence differences [1,2], it was suggested recently to re-name plant Rac GTPases as a distinct family called Rop (Rho-related GTPase from plants). Both terms, Rac and Rop, are currently used in the literature to identify the same type of small plant GTPase [6,7].

Rac/Rop GTPases in plants are involved in multiple developmental processes [1,2]. The *Arabidopsis* genome contains at least 11 Rac/Rop GTPases [8]. *At*Rac1, also called *At*ROP6, is involved in stomatal closure [6], and *Os*Rac1 from rice is associated with reactive oxygen species (ROS) production [5,9]. *At*ROP1, 3, and 5 GTPases are functionally redundant, and are involved in establishing cell polarity in growing pollen tubes [2,10,11]. Other members of the subfamily, *At*ROP2, 4, and 6, are involved in root hair growth [12,13]. The common mechanism of action for these Rop GTPases is believed to be in controlling cell growth by regulating the dynamic assembly of cortical F-actin [14] or by controlling actin binding and depolymerizing activity of actin-depolymerizing factors [7]. Recent evidence suggests that *At*ROP2 may activate the actin-nucleating BRICK1/ARP2/3 pathway leading to localized patches of actin in certain epidermal cells to influence morphology in diffusely growing tissues [14].

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A central interest of our laboratory has been monitoring changes in the cytoskeleton during cotton fiber development [15–19]. Due to their highly elongated structure, high cellulose content, and lack of cell division, cotton fibers are a good experimental model for studying the relationship between the cytoskeleton and plant cell wall [20]. Since the fiber is elongating from the day of anthesis (DOA) to approximately 21–26 days post-anthesis (DPA) by a combination of tip growth and polar diffuse growth [21], a thin primary wall delimits the cell. Fiber secondary wall synthesis is initiated approximately 15–16 DPA, overlapping the final stages of fiber elongation. In the transition from cell elongation to the onset of secondary cell wall synthesis, there are dramatic changes; microtubules increase in number, length, and proximity to the plasma membrane, cortical microtubule orientation is changed from transverse to steeply pitched helices [22], the rate of cellulose synthesis is estimated to rise nearly 100-fold in vivo and the synthesis of other cell wall polymers ceases [23].

Two cotton Rac/Rop GTPases, *GhRac9* and *GhRac13*, were previously reported to be expressed at low levels during cell elongation and highly expressed during the transition period prior to secondary cell wall synthesis [4]. Due to the timing of their expression, it is unlikely that *GhRac9* or *GhRac 13* are involved in fiber elongation. The constitutively active form of *Gh*Rac13 induced ROS in cultured cells of soybean and *Arabidopsis* [24,25]. When an inhibitor of NADPH oxidase, diphenyleneidonium (DPI), was added to cotton ovule cultures, H_2O_2 production was inhibited and secondary wall synthesis was delayed. In mammals, Rac GTPases control production of ROS by regulating the activity of plasma membrane associated NADPH oxidase complexes [26]. Thus, it was hypothesized that *Gh*Rac13 GTPase might regulate ROS production through the plasma membrane NADPH oxidase complex [24,25] in a manner similar to constitutively active *Os*Rac1-induced H_2O_2 production in rice leaf intercellular spaces [5].

Previously, we isolated *GhRac1*, a member of Rac/Rop GTPase, from 5 DPA cottonseeds with associated fibers [27]. Due to the sequence similarity between *GhRac13* and *GhRac1* we were uncertain whether these two genes were homologues, homeologues, or related members of a small gene family. Here, we characterize *Gh*Rac1 and compare its developmental and tissue-specific expression patterns with *GhRac13*. In contrast to *GhRac9* and *13*, *GhRac1* was highly expressed during fiber elongation and minimally expressed when secondary cell wall synthesis began. This pattern of expression is similar to several other genes that are required for fiber elongation [17,28,29]. *GhRac1* was also abundantly expressed in other elongating tissues, but *GhRac13* was not. The expression pattern of *GhRac1* suggests that it may be involved in cytoskeleton dynamics in elongating fibers as well as other elongating tissues.

2. Materials and methods

2.1. Plant materials

Immature seeds with fiber (*Gossypium hirsutum* cv. Texas Marker 1) were harvested from plants by 9 AM and were frozen in liquid nitrogen. Developing ovaries were collected at 2-day intervals from 8 through 20 DPA. Fibers were carefully scraped from the frozen ovules using a scalpel.

Mature leaves (15 cm in diameter), young leaves (5 cm in diameter), hypocotyls and roots were harvested from 1- or 6-week-old plants grown in a greenhouse at 25–32 °C. All tissues were frozen in liquid nitrogen, and stored at −80 °C.

2.2. Cloning and sequencing of GTPases

A full-length cDNA of *GhRac1* (AF165925) from *G. hirsutum* cv. Texas Marker 1 was previously obtained using RT-PCR and 5′ RACE [27]. To compare the expression pattern of *GhRac1* with *GhRac13*, *GhRac13* containing coding sequences and 3′ untranslated region (UTR) from *G. hirsutum* cv. Texas Marker 1 was amplified by 3′ RACE (Clontech, Palo Alto, CA) using primers designed from *GhRac13* (S79308) from *G. hirsutum* cv. Coker 130. Amplified products were cloned in the plasmid PCR II (Invitrogen, Carlsbad, CA) and sequenced using the 4000L Automated DNA sequencer (LI-COR, Lincoln, NE) with the cycle sequencing protocol from the SequiTherm EXCEL II Long-Read Sequencing Kit-LC (Epicentre Technologies, Madison, WI).

2.3. Real-time quantitative RT-PCR

Primers of *GhRac1* (5′ -GAAATGGATTCCAGATTTGAGACA-3′ /5′ -AAGATCAAGCTTAGGCCCAACA-3′) and *GhRac13* (5′ GTGAAGGCTGTTTTCGAT GCT-3′ / 5′ -TTTTGCAAGGCTTTCTCTTTGG-3′) were designed using Primer Express® software (version 2.0, Applied Biosystems, Foster City, CA). The specificity of primer annealing was examined by monitoring the dissociation curve when real-time PCR reactions were completed. Cotton *α-tubulin4* (5′ -GATCTCGCTGCCCTGGAA-3′ / 5′ -ACCAGACTCAGCGCCAACTT-3′), expressed constitutively during fiber development [17] and cotton *18S rRNA* (5′ -CGTCCCTGCCCTTTGTACA-3′ /5′ -AACACTTCACCGGACCATTCA-3′) were used as normalizers. The amplicon sizes of *GhRac1* (73 base pairs), *GhRac13* (73 base pairs), *α-tubulin4* (51 base pairs) and *18S rRNA* (61 base pairs) were designed to be less than 150 base pairs to make amplification efficiencies equivalent.

Total RNAs from cotton tissues of different developmental stages were isolated according to the method of Schultz et al., [30] treated with DNase I (Ambion, Austin, TX) before cDNA amplification. First-strand

complementary DNA was synthesized using 1 μg of total RNA by priming with random hexamers at 48 °C for 30 min followed by inactivation of MultiScribe™ Reverse Transcriptase (Applied Biosystems) at 95 °C for 10 min. Real-time RT-PCR was performed using the SYBR® Green PCR Master Mix in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s for denaturation and 60 °C for 1 min for annealing and extension. Reported values are the average of triplicate PCR reactions. The experiments were repeated twice independently beginning with RNA isolation.

Amplification efficiencies among samples varied by less than 0.01 for each cycle of amplification. Relative transcript levels were determined by a comparative C_T method according to Applied Biosystems' guidelines [37]. Statistical analyses and construction of graphs were performed using Prism version 4.00 software.

2.4. Genomic Southern blot analysis

Genomic DNA from TM 1 ovules (DOA) was isolated by Plant DNAzol (Life Technologies, Grand Island, NY). Four micrograms of genomic DNA were digested with restriction enzymes and separated on a 0.7% agarose gel. The DNA was transferred to a BrightStar nylon membrane (Ambion) with 5× SSC and 400 mM NaOH. Specific probes for *GhRac1* and *GhRac13* were amplified from 3′ UTRs. The 3′ UTR of *GhRac1* was amplified with a primer set (5′ -GTAGTTCTCCAGCCTCCAAAGAAG-3′ /5′ -CCCTAGTTCTGTCAGTTCAGATAAC-3′) and the 3′ UTR of *GhRac13* was amplified with a primer set (5′ -GTAGCTTTGAGGCCACCAAAACCA-3′ /5′ -CGATTCATTGATCGATGGGTTGAT-3′). Probes were labeled with [^{32}P]-dCTP (3000 Ci/mmol) by random primer (Ambion). The membranes were hybridized with the radiolabeled DNA at 42 °C, then washed at 60 °C in 0.1× SSC and 0.1% SDS, and autoradiographed.

2.5. Construction of recombinant GhRac1

The coding region of *GhRac1* cDNA (AF165925) was PCR-amplified by primers (5′ -AAGAATTCATGAGTGCTTCCAGGTTCATAA-3′ /5′ -TTGTCGACCAATATTGAGCAA GCTTTTTGTGCC-3′) and ligated in-frame between the *Eco*RI and *Sal*I sites of pET-29a(+) (Novagen, Madison, WI), a plasmid used for expression of 6× His-tagged proteins. The recombinant plasmid was transformed into BL21 (DE3) cells containing pLysS. The protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. The 6× His-tagged recombinant protein, expressed in the bacterial soluble fraction, was purified on a nickel affinity column (Novagen). Purified recombinant protein from bacteria was separated on a 12.5% SDS polyacrylamide gel.

2.6. GTPase assay

The purified recombinant *Gh*Rac1 was dialyzed with 20 mM Tris, pH 8.0 and 200 mM NaCl. For the assay of GTPase activity, purified recombinant *Gh*Rac1 in 20 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, and 100 mM NaCl was incubated for 30 min with 3 mM GTP at 37 °C. After addition of the Ames reagent [31], samples were incubated for 30 min at 45 °C. Inorganic phosphate produced by *Gh*Rac1 GTPase was measured at A_{820} against the blank buffer containing no *Gh*Rac1 [31].

3. Results and discussion

3.1. Phylogenetic analysis of Rop GTPases expressed in cotton fibers

Analysis of the *Arabidopsis* genome revealed that there are at least 11 *Arabidopsis* Rac GTPases that can be classified into two distinct groups [8]. More recent structural and functional analyses of small GTPases in *Arabidopsis* led to the suggestion of re-naming Rac GTPases as Rop GTPases. The Rop GTPases are classified into four subfamilies by sequence analyses with Clustal W and PAUP [1,2].

Our phylogenetic comparison of Rop GTPases from *Arabidopsis* and Rac GTPases from cotton and rice was performed using MAFFT [32] and also led to the classification of four subfamilies (Fig. 1). Group I contains

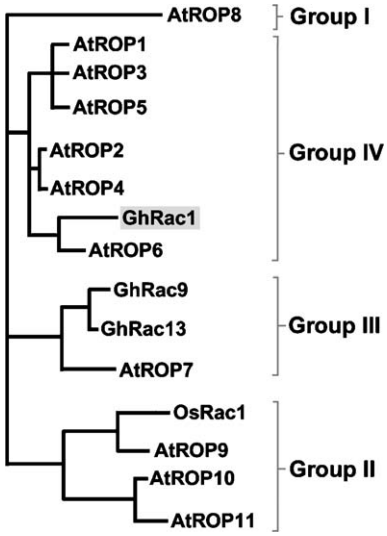

Fig. 1. Phylogenetic tree of Rac/Rop GTPases. Amino acid sequences were analyzed by MAFFT (Katoh et al. [32]). *At*ROP1 (*Arabidopsis thaliana*, AAC78390); *At*ROP2 (AAC78391); *At*ROP3 (AAF40237); *At*ROP4 (AAF40244); *At*ROP5 (AAF40245); *At*ROP6 (AAF40242); *At*ROP7 (AAF40241); *At*ROP8 (AAD42972); *At*ROP9 (AAF40246); *At*ROP10 (AAF40247); *At*ROP11 (BAB10857); *Gh*Rac1 (*G. hirsutum*, AAD47828); *Gh*Rac9 (Q41254); *Gh*Rac13 (Q41253); and *Os*Rac1 (*Oryza sativa*, BAA84492).

only *At*Rop8, the function of which is unknown. Group II contains *Os*Rac1 that is involved in H_2O_2 production [5] and *At*Rop 9, 10, and 11, two of which (*At*Rop9 and 10) are involved in ABA responses [33]. Group III of the *Arabidopsis* Rop GTPase family contains only *At*Rop7, which appears to inhibit root hair tip growth [13]. Group IV is the largest group with *At*ROP1 to 6, all of which are involved in pollen tube and root hair growth [12–14], or stomatal closure [6] by mediating the actin cytoskeleton.

The three fully sequenced cotton Rac/Rop GTPase family members are *Gh*Rac1 (AF165925), *Gh*Rac9 (S79309), and *Gh*Rac13 (S79308). In the phylogenic tree generated by MAFFT, *Gh*Rac1 is a member of group IV, whereas *Gh*Rac9 and 13 are members of group III (Fig. 1).

3.2. Southern blot analysis of GhRac1 and GhRac13

GhRac1 GTPase cDNA (AF165925) was isolated from immature cottonseeds (*G. hirsutum* cv. Texas Marker 1, 6 DPA) by RT-PCR and RACE PCR [27]. The open reading frame of *Gh*Rac1 consists of a 198 amino acid polypeptide in which the GTPase domains (8–23, 56–65), effector domain (25–53), GDP/GTP binding domains (113–121, 155–161), Rho insert region (125–135), two putative serine/theronine-dependent phosphorylation sites (74–76, 159–161), and a consensus carboxyl motif CAAL (C, cysteine; L, leucine; A, aliphatic amino acids) for geranygeranylation [34] are found. The DNA sequences of *GhRac1* isolated from *G. hirsutum* cv. Texas Marker 1 and *GhRac13* from *G. hirsutum* cv. Coker 130 were very similar except in the 3′ UTR (Fig. 2).

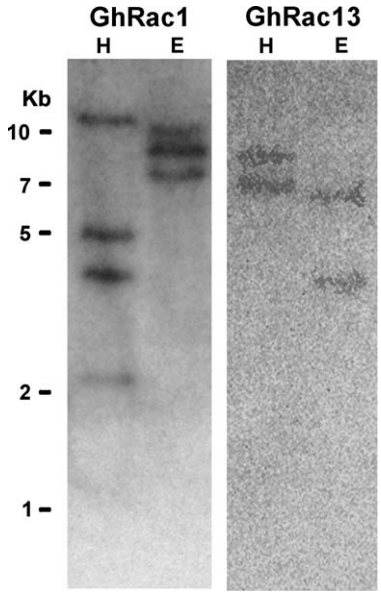

Fig. 3. Southern blot analysis of *GhRac1* and *GhRac13* genomic sequences. Cotton genomic DNA was digested with *Hin*dIII (H) and *Eco*RI (E). Gene-specific probes of either *GhRac1* or *GhRac13* were made by random priming with [^{32}P]-dCTP. The blot was hybridized at 45 °C, washed with 0.1×SSC and 0.1×SDS at 60 °C, and autoradiographed.

To determine whether *GhRac1* is a different gene from *GhRac13*, we first isolated *GhRac13* from Texas Marker 1 by 3′ RACE PCR. The coding and UTR sequences of *GhRac13*

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GhRac1     ----MSASRFIKCVTVGDGAVGKTCLLISYTSNTFPTDYVPTVFDNFSANVVVDGNTVNL 56
AtROP6     ----MSASRFIKCVTVGDGAVGKTCLLISYTSNTFPTDYVPTVFDNFSANVIVDGNTINL 56
GhRac13    ----MSTARFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGSTVNL 56
GhRac9     ----MNTSRFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGSTVNL 56
OsRac1     MSSAAAATRFIKCVTVGDGAVGKTCMLICYTCNKFPTDYIPTVFDNFSANVSVDGSVVNL 60

GhRac1     GLWDTAGQEDYNRLRPLSYRGADVFLLAFSLISKASYENVAKKWIPELRHYAPGVPIILV 116
AtROP6     GLWDTAGQEDYNRLRPLSYRGADVFLLAFSLVSKASYENVSKKWVPELRHYAPGVPIILV 116
GhRac13    GLWDTAGQEDYNRLRPLSYRGADVFLLAFSLISKASYENIYKKWIPELRHYAHNVPVVLV 116
GhRac9     GLWDTAGQEDYNRLRPLSYRGADVFLLAFSLISKASYENVHKKWIPELRHYAPNVPIVLV 116
OsRac1     GLWDTAGQEDYSRLRPLSYRGADVFILSFSLISRASYENVQKKWMPELRRFAPGVPVVLV 120

GhRac1     GTKLDLREDKQFFIDHPGAVPITTAQGEELRKLIGAHFYIECSSKTQQNVKAVFDAAIKV 176
AtROP6     GTKLDLRDDKQFFAEHPGAVPISTAQGEELKKLIGAPAYIECSAKTQQNVKAVFDAAIKV 176
GhRac13    GTKLDLRDDKQFLIDHPGATPISTSQGEELKKMIGAVTYIECSSKTQQNVKAVFDAAIKV 176
GhRac9     GTKLDLRDDKQFLSDNPGAISITTSQGEELKKMVGAVTYIECSSKTQQNVKVVFDVAIKI 176
OsRac1     GTKLDLREDRAYLADHPASSITTEQGEELRKLIGAVAYIECSSKTQRNIKAVFDTAIKV 180

GhRac1     VLQPPK-KKKKKKRKAQKACSIL-------------- 198
AtROP6     VLQPPK-NKKKKKRKSQKGCSIL-------------- 198
GhRac13    ALRPPK-PKRK-PCKRRT-CAFL-------------- 196
GhRac9     ALRPPK-PKRK-PIKRRS-CAFL-------------- 196
OsRac1     VLQPPRHKDVTRKKLQSSSNRPVRRYFCGSACFA--- 214
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Fig. 2. Multiple alignment of Rac/Rop deduced amino acid sequences. Identical residues are highlighted. GTPase domains (8–23, 56–65 of *Gh*Rac1) are underlined. The carboxyl terminal motif (195–198 of *Gh*Rac1) for geranygeranylation is indicated by bold font.

from Texas Marker 1 were identical to those of *GhRac13* from Coker 130 and the 3′ UTR sequences of *GhRac1* and *GhRac13* show low sequence similarity. Thus, the 3′ UTR sequences of both *GhRac1* and *GhRac13* were used as gene-specific probes for Southern blot analysis. There are two copies of *GhRac13* in the Coker 130 genome [4]. Similarly, two bands were detected in Texas Marker 1 genomic DNA digested by *Hin*dIII or *Eco*RI (Fig. 3). Southern blot analysis of *GhRac1* further confirms that *GhRac1* is a member of a multigene family and is different from *GhRac13*.

3.3. Developmental expression of GhRac1 and GhRac13 during fiber development

Since *GhRac13* is known to be fiber-specific and developmentally regulated [4], we have examined if *GhRac1* expression is regulated during fiber development. Real-time RT-PCR was conducted with SYBR® Green PCR Master Mix and gene-specific PCR primers. DNA contamination in purified cotton RNA was eliminated by DNase I digestion before cDNA synthesis with random hexamers. Primer specificity was confirmed by analyzing dissociation curves of the PCR amplification products [37]. Transcript levels of *GhRac1* and *13* were normalized with respect to those of *α-tubulin4*, a fiber-specific gene that is expressed constitutively throughout fiber development [17]. For specific amplification of *α-tubulin4*, the primers were designed from the 3′ UTR sequence. After conducting numerous analyses of fiber-specific genes, we have found that *α-tubulin4* is a better normalizer for developmentally regulated genes during fiber development than *18S rRNA* or *actin,* two genes commonly used for real-time PCR (unpublished results).

GhRac1 and *GhRac13* are both expressed in cotton fibers and exhibit developmentally regulated patterns of expression during development. The expression patterns of the two genes are quite distinct. Transcript levels of *GhRac13* peaked during the transition period (14–16 DPA) and are six times higher than levels during elongation (10 DPA) and secondary (20 DPA) wall synthesis stages. The transcript levels of *GhRac1* peaked during the elongation stage (8–14 DPA), and declined coincident with the initiation of secondary wall synthesis (16 DPA) (Fig. 4A). Transcript abundance of *GhRac1* in 10 DPA fibers was 14 times higher than in 20 DPA fibers. The expression pattern of *GhRac1* is identical to those of other genes expressed primarily during the cell elongation stage of fiber development [17,28,29].

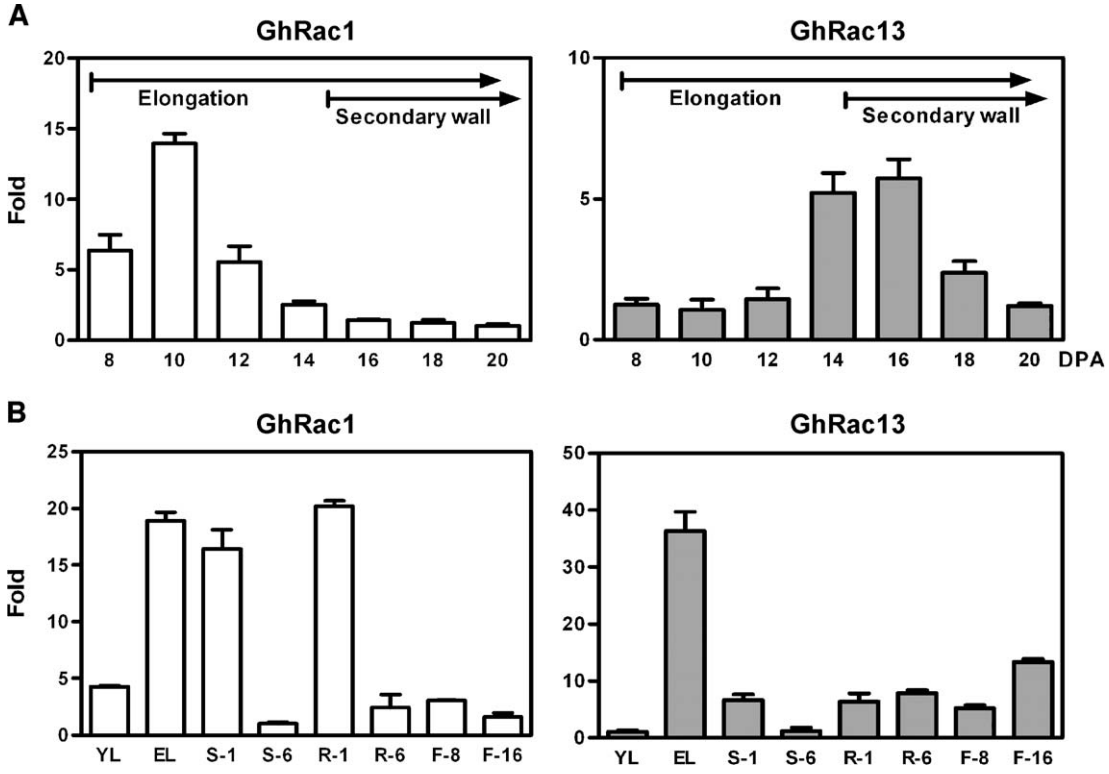

Fig. 4. Developmental expression of *GhRac1* and *GhRac13* in fiber tissues harvested throughout the transition from cell elongation to secondary wall synthesis (A). Real-time RT-PCR was performed using the SYBR® Green PCR Master Mix in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with specific primers for *GhRac1* and *13*. The transcript levels of *GhRac1* and *13* were normalized with respect to the transcript level of *α-tubulin4*. (B) Preferential expression of *GhRac1* and *13* in other cotton tissues. DNA-free total RNAs were extracted from young leaves (YL), fully expanded leaves (EL), 1-week-old hypocotyls (S-1), 6-week-old stems (S-6), 1-week-old roots (R-1), 6-week-old roots (R-6), 8 DPA fibers (F-8), and 16 DPA fibers (F-16). Real-time RT-PCR analyses were conducted as described in panel A. The transcript levels of *GhRac1* and *13* were normalized with respect to the transcript level of *18S* ribosomal RNA.

3.4. Tissue-specific expression of GhRac1 and GhRac13

To compare the tissue-specific expression patterns of *GhRac1* with *GhRac13*, real-time RT-PCR was performed using RNA isolated from young expanding and fully expanded leaves, 1-week-old hypocotyls, 6-week-old stems, and 1- and 6-week-old roots in addition to developing fibers. Since *α-tubulin4* is preferentially expressed in cotton fibers and not expressed in other tissues [17], *18S rRNA* had to be used as a normalizer for this experiment.

GhRac13 was reported to be a fiber-specific gene since it was not detected by Northern blot analysis of seedling leaves and roots or flowers [4]. In contrast, real-time RT-PCR analysis clearly shows that *GhRac13* was expressed in all tested cotton tissues (Fig. 4B). The difference may be explained by the increased sensitivity of real-time RT-PCR as an analytical method for measuring RNA abundance. In young leaves and 6-week-old stems, *GhRac13* showed the lowest expression level (1×). The highest levels (36×) were found in fully expanded leaves, a tissue that had not been tested in the earlier study. In both hypocotyls and roots, *GhRac13* was expressed as abundantly as in young cotton fibers (Fig. 4B).

In contrast to *GhRac13*, *GhRac1* shows a high level of expression in elongating tissues such as 1-week-old hypocotyls (16×), 1-week-old roots (20×), and fully expanded leaves (19×). Lower levels of *GhRac1* transcripts were found in 6-week-old stems (1×) and 6-week-old roots (2×) (Fig. 4B).

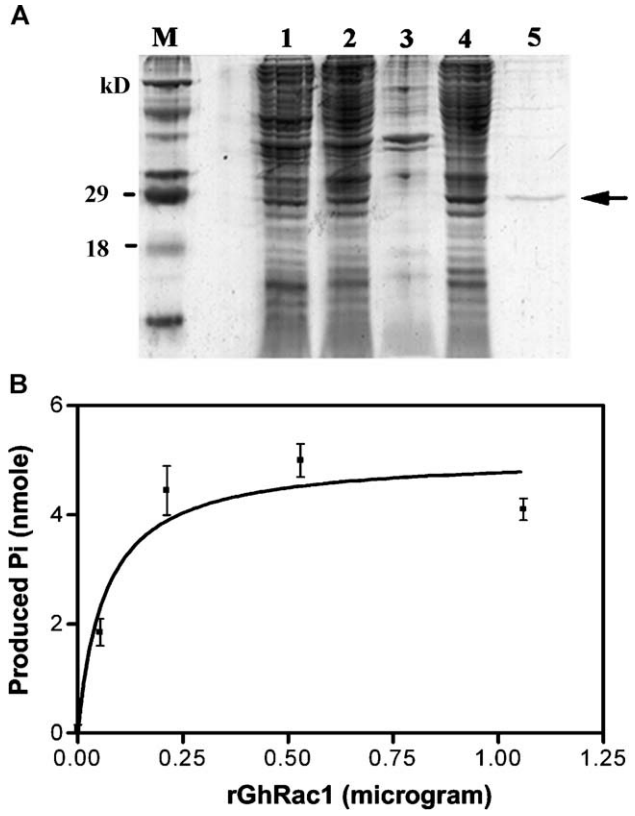

Fig. 5. Purification of recombinant *Gh*Rac1 and GTPase assay. (A) The 6× His-tagged recombinant *Gh*Rac1 protein was expressed in *E. coli*, purified on a nickel column and electrophoresed on a 12.5% acrylamide SDS gel stained with Coomassie blue. Lane 1, uninduced lysate; lane 2, induced lysate; lane 3, insoluble proteins of lysate; lane 4, soluble proteins of lysate; lane 5, purified recombinant *Gh*Rac1. (B) Inorganic phosphate hydrolyzed from GTP by recombinant *Gh*Rac1 GTPase (r*Gh*Rac1) was stained with Ames reagent, and then detected spectrophotometrically at 820 nm.

3.5. GhRac1 encodes a functional GTPase

The recombinant *Gh*Rac1 protein, containing an S-tag at the N-terminus and 6× His-tag at the C-terminus, was expressed in *E. coli*. Recombinant *Gh*Rac1 protein (r*Gh*Rac1) was soluble in the presence of 100 mM NaCl, and was purified on a nickel affinity column. Affinity purified r*Gh*Rac1 was separated on a 12.5% SDS polyacrylamide gel and resulted in a single protein. The open reading frame of *GhRac1* consists of a 198 amino acid polypeptide of 21.8 kDa. The predicted molecular mass of the r*Gh*Rac1 with both protein tags was 27.3 kDa, consistent with the MW determined by SDS-PAGE (Fig. 5A).

In plants, directional cell expansion may occur by either tip growth or polar diffuse growth [35]. The unidirectional growth of pollen tubes and root hairs depends on tip growth where expansion is driven by fusion of post-Golgi vesicles to a specific region of the plasma membrane. In contrast, cells that expand by polar diffuse growth, such as cotton fiber cells, have been hypothesized to be constrained by the organization and mechanical properties of cell wall components, especially cellulose microfibrils, which may limit the direction of cell expansion. Recent evidence suggests that this model for polar diffuse growth is not complete [36]. Rac/Rop GTPases have been proposed to control the direction of cell expansion through the regulation of the actin cytoskeleton [7,14]. Specific point mutations in GTPases can produce constitutively active (GTP-locked form to activate Rac/Rop-dependent signaling) or dominant-negative (GDP-locked form to inactive Rac/Rop-dependent signaling) forms of the enzymes. The constitutively active form of *At*ROP6, the member of group IV most similar to *Gh*Rac1, causes loss of tip growth resulting in root hair swelling [12].

GhRac1 in cotton is abundantly expressed in rapidly elongating tissues such as hypocotyls, young roots, and elongating fibers. *GhRac1* is transiently expressed during the elongation stage of cotton fiber development. The temporal expression pattern distinguishes *GhRac1* from previously identified cotton Rac/Rop GTPases, *GhRac9* and *GhRac13*, that are expressed during the transition to secondary cell wall production. *GhRac13* was suggested to be involved in secondary wall synthesis by mediating H_2O_2 production through NADPH oxidase since over-expression of *GhRac13* induced ROS in cultured soybean

and *Arabidopsis* cells [24,25]. *GhRac1* belongs to Rac/Rop group IV, whose members are involved in tip growth and cell expansion of pollen tubes [10,11] or root hairs [12,13]. Recently, it was shown that *AtROP2*, a member of Rac/Rop group IV, could modulate both polar diffuse growth and tip growth [14].

The association of maximal *GhRac1* expression with stages of maximal cotton fiber elongation suggests that *Gh*Rac1 is important for cell wall expansion. Our current working hypothesis is that *Gh*Rac1 GTPase is involved in cytoskeletal assembly during the fiber cell elongation stage, similar to the role of other group IV Rac/Rop GTPases. We are constructing constitutively active and dominantly negative forms of *GhRac1* to test this hypothesis directly.

Acknowledgements

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